staining was observed in the multinucleated cells, indicating that osteoclast-like giant cells were dominant on the compression side (Fig. 3*B*). In contrast, the localization of osteocalcin (*Ocn*) mRNA-positive cells was observed in the cells on the tension side, indicating that osteoblast-like cells were dominant (Fig. 3*B*). A fluorescent double-labeling experiment using calcein and tetracycline further showed that incorporation of these reagents into the alveolar bone on the tension side, but not the compression side, was clearly observable in the double-labeled line within 10 days of the orthodontic treatment (Fig. 3*C*). These findings suggest that the PDL of the bioengineered tooth successfully mediates bone remodeling via the proper localization of osteoclasts and osteoblasts in response to mechanical stress.

Perceptive Potential of Neurons Entering the Tissue of the Bioengineered Tooth. The perception of noxious stimulations such as mechanical stress and pain, are important for the protection and proper functions of teeth (34). Neurons in the trigeminal ganglion, which innervate the pulp and PDL, can detect these stress events and transduce the corresponding perceptions to the central nervous system (34). We have previously reported that nerve fibers are detectable in the pulp of a developing bioengineered tooth in the oral cavity (29). In our current experiments, we evaluated the responsiveness of nerve fibers in the pulp and PDL of the bioengineered tooth to induced noxious stimulations.

Anti-neurofilament (NF)-immunoreactive nerve fibers were detected in the pulp, dentinal tubules, and PDL of the bioengineered tooth as in a normal tooth (Fig. 4*A* and Fig. S4). Neuropeptide Y (NPY), which is synthesized in sympathetic nerves (34), was also detected in the pulp and PDL neurons (Fig. 4*A* and Fig. S4 *C* and *D*). Calcitonin gene-related peptide (CGRP), which is synthesized in sensory nerves and is involved in sensing tooth pain (34) was also observed in both pulp and PDL neurons (Fig. 4*A* and Fig. S4 *E* and *F*). NPY and CGRP were detected in both the anti-NF positive and negative-immunoreactive neurons (Fig. 4*A* and Fig. S4 *C–F*).

We next evaluated the perceptive potential of these neurons in the bioengineered tooth against noxious stimulations such as orthodontic treatment and pulp stimulation. The expression of galanin, which is a neuropeptide involved in pain transmission (35), increased in response to persistent painful stimulation of the nerve terminals within the PDL of the bioengineered tooth to the same extent as in a normal tooth (Fig. 4*B*). Thus PDL nerve fibers in the bioengineered tooth appear to respond to nociceptive stimulation caused by our experimental tooth movements. Previous studies have reported that neurons expressing the proto-oncogene c-Fos protein are detectable in the superficial layers of the medullary dorsal horn following noxious stimulations such as electrical, mechanical and chemical stimulation of intraoral receptive fields involving the tooth pulp, PDL, and peripheral nerves innervating the intraoral structures (34, 35). We found in our current analyses that the c-Fos-immunoreactive neurons present in both the normal tooth and the bioengineered tooth drastically increased at 2 h after experimental tooth movement, and then gradually decreased within 48 h (Fig. 4*C*). Following pulp stimulation, positive neurons in both normal and bioengineered teeth also increased at 2 h after stimulation, but could not be detected at 48 h (Fig. 4*D*). These data indicate that the nerve fibers innervating both the pulp and PDL of the bioengineered tooth have perceptive potential for nociceptive stimulations and can transduce these events to the central nervous system (the medullary dorsal horn).

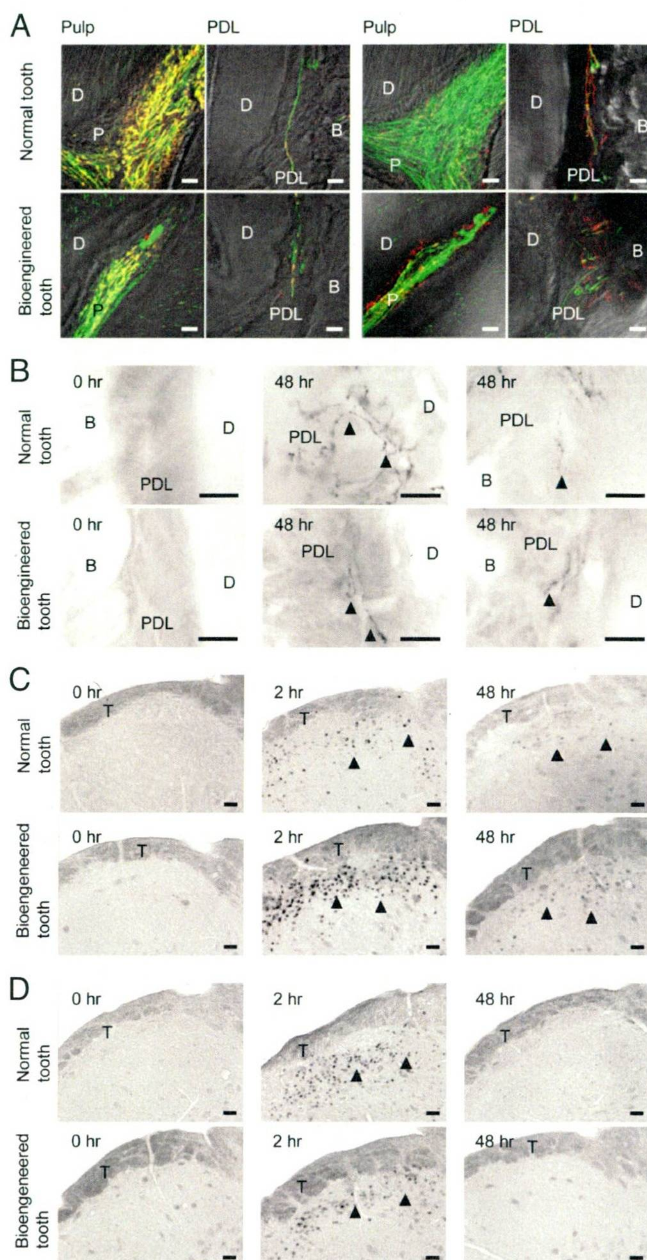

Fig. 4. Pain response to mechanical stress. (*A*) Nerve fibers in the pulp and PDL in the normal (*Upper*) and bioengineered (*Lower*) tooth were analyzed immunohistochemically using specific antibodies for the combination (left 2 columns) of NF (green) and NPY (red) and the combination (right 2 columns) of NF and CGRP (red). (Scale bar, 25 μm.) (*B*) Analysis of galanin immunoreactivity in the PDL of a normal (*Upper*) and bioengineered (*Lower*) tooth for the assessment of orthodontic force. No galanin expression was evident in the untreated tooth (*Left*). Galanin expression (arrowhead) was detected in the PDL of a normal and bioengineered tooth after 48 h of orthodontic treatment (*Right*). (Scale bar, 25 μm.) (*C*) Analysis of c-Fos-immunoreactivity in the medullary dorsal horn of mice with a normal tooth (*Upper*) or a bioengineered tooth (*Lower*) after 0 h (*Left*), 2 h (*Center*), and 48 h (*Right*) of orthodontic treatment. c-Fos expression (arrowhead) was also detected. (Scale bar, 50 μm.) (*D*) Analysis of c-Fos immunoreactivity in the medullary dorsal horn of mice with a normal tooth (*Upper*) or a bioengineered tooth (*Lower*) after 0 h (*Left*), 2 h (*Center*), and 48 h (*Right*) of stimulation by pulp exposure. c-Fos expression (arrowhead) was evident in the medullary dorsal horn after 2 and 48 h of pulp exposure. (Scale bar, 50 μm.) D, dentin; P, pulp; B, bone; PDL, periodontal ligament; T, spinal trigeminal tract.

Discussion

We successfully demonstrate herein that our bioengineered tooth germ develops into a fully functioning tooth with sufficient hardness for mastication and a functional responsiveness to mechanical stress in the maxillofacial region. We also show that the neural fibers that have re-entered the pulp and PDL tissues of the bioengineered tooth have proper perceptive potential in response to noxious stimulations such as orthodontic treatment and pulp stimulation.

These findings indicate that bioengineered tooth generation techniques can contribute to the rebuilding of a fully functional tooth.

Critical issues in tooth regenerative therapy are whether the bioengineered tooth can reconstitute functions such as mastication (32) and responsive potential to mechanical stress (31, 33) and noxious stimulations (34), including cooperation of the regenerated tooth with both the oral and maxillofacial regions. Eruption and occlusion are essential first steps toward dental organ replacement therapy and successful incorporation into the oral and maxillofacial region (21, 36). Our laboratory has demonstrated previously that a bioengineered tooth germ can develop into a tooth with the correct structure in an adult mouse (29). It has also been reported previously that normal tooth germ isolated from murine embryos and a bioengineered tooth constructed from cultured tooth bud cells can develop and erupt in a toothless oral soft tissue region (diastema) of adult mice and in the tooth extraction sockets of an adult rat (37–42). In our current study, we provide evidence that a bioengineered tooth with the same hardness as an adult natural tooth can erupt with normal gene expression, including *Csf1* and *Pthr1*, which are thought to regulate osteoclastogenesis, and achieve functional occlusion with the opposing natural teeth. Previous reports have suggested that the eruption of tooth germ is generally induced at the site of tooth development and by the gubernacular cord, which is derived from the epithelium of the dental lamina (43). Hence, our findings provide significant insights into tooth eruption mechanisms and strongly suggest that masticatory potential can be successfully restored by the transplantation of bioengineered tooth germ.

To establish cooperation between the bioengineered tooth and the maxillofacial region, 1 critical issue to address is whether a functional PDL is achieved and thereby the restoration of interactions between the bioengineered tooth and the alveolar bone (31, 33). The PDL has essential roles in tooth support, homeostasis, and repair, and is involved in the regulation of periodontal cellular activities such as cell proliferation, apoptosis, the secretion of extracellular matrices, the resorption and repair of the root cementum, and remodeling of the alveolar bone proper (31, 33). Although implant therapy has been established and is effective for replacement of a missing tooth, this therapy involves osseointegration into the alveolar bone that does not reconstitute the PDL (44). The regeneration of PDL has been studied previously using cell sheets (17) and stem cells (22), but has not yet been fully successful. It is thought that orthodontic tooth movement, a process involving pathogenic and physiologic responses to extreme forces applied to a tooth through bone remodeling controlled by osteogenesis and osteoclastgenesis (31), is a good assay model for the evaluation of PDL functions. In our present study, the PDL associated with the bioengineered tooth performed in complete cooperation with the oral and maxillofacial regions and bone remodeling successfully occurred following the application of orthodontic mechanical force. These findings indicate that it is possible to restore and re-establish cooperation between the bioengineered tooth and maxillofacial regions and thus regenerate critical dental functions.

The peripheral nervous system plays important roles in the regulation of organ functions and the perception of external stimuli such as pain and mechanical stress (45). During development of the peripheral nervous system, growing axons navigate and establish connections to their developing target organs (46). The recovery of the nervous system, which is associated with the reentry of nerve fibers, is critical for organ replacement (47). Although the functions of several internal organs, including the liver, kidney, and pancreas, are also mediated by specific humoral factors such as hormones and cytokines via blood circulation (45), perceptions of external stimuli are also essential to the functions of several organs, such as the eye, limbs, and teeth (45). The tooth is well recognized as a peripheral target organ for sensory trigeminal nerves, which are required for the function and protection of the teeth (46). It is known also that the perception of mechanical forces during mastication is limited in implant patients (48). Thus, the restoration of nerve functions is also critical for tooth regenerative therapy and future organ replacement therapy (13, 45). In our current study, we demonstrate that several species of nerve fibers, including NF, NPY, CGRP, and galanin-immunoreactive neurons, successfully re-entered both the pulp and/or PDL region of the bioengineered tooth. These nerves could thereby transduce the signals from noxious stimulations such as mechanical stress by orthodontic treatment and the exposure of pulp. Previous studies have also revealed that trigeminal nerve fibers navigate and establish their axonal projections into the pulp and PDL during early tooth development in a spatiotemporally controlled manner through expression of regulatory factors such as nerve growth factor, glial cell line-derived neurotrophic factor, and semaphorin 3a (46). Our present results suggest the possibility that the transplantation of regenerated tooth germ can induce trigeminal axon innervation and establishment in an adult jaw through the replication of trigeminal axon pathfinding and nerve fiber patterning during early tooth development (46).

In conclusion, this study provides evidence of a successful replacement of an entire and fully functioning organ in an adult body through the transplantation of bioengineered organ germ, reconstituted by single cell manipulation in vitro. Our study therefore makes a substantial contribution to the development of bioengineering technology for future organ replacement therapy. Further studies on the identification of available adult tissue stem cells for the reconstitution of a bioengineered tooth germ and the regulation of stem cell differentiation into odontogenic cell lineage will help to achieve the realization of tooth regenerative therapy for missing teeth.

Methods

Transplantation. The upper first molars of 5-week-old C57BL/6 (SLC) mice were extracted under deep anesthesia. Mice were maintained for 3 weeks to allow for natural repair of the tooth cavity and oral epithelium. Before transplantation, we confirmed using microCT analysis that the remaining tooth root components and/or the tooth that had developed from them could not be observed in the bony holes (*SI Methods*). Following repair, an incision of approximately 1.5 mm in length was made through the oral mucosa at the extraction site with fine scissors to access the alveolar bone. A fine pin vice (Tamiya) was used to create a bony hole of about 0.5–1.0 mm in diameter in the exposed alveolar bone surface. Just before transplantation, we removed the collagen gel from the bioengineered tooth germ in the in vitro organ culture and marked the top of the dental epithelium with vital staining dye, such as methylene blue, to ensure the correct direction of the explants. The explants were then transplanted into the bony hole according to the dye. The incised oral mucosa was next sutured with 8–0 nylon (8–0 black nylon 4 mm 1/2R, Bear Medic Corp.) and the surgical site was cleaned. The mice containing the transplants were fed a powdered diet (Oriental Yeast) and skim milk until the regenerated tooth had erupted.

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MEDICAL SCIENCES

1. Brockes JP, Kumar A (2005) Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* 310:1919–1923.
2. Watt FM, Hogan BL (2000) Out of Eden: Stem cells and their niches. *Science* 287:1427–1430.

3. Langer RS, Vacanti JP (1999) Tissue engineering: The challenges ahead. *Sci Am* 280:86–89.
4. Atala A (2005) Tissue engineering, stem cells and cloning: Current concepts and changing trends. *Expert Opin Biol Ther* 5:879–892.
5. Korbling M, Estrov Z (2003) Adult stem cells for tissue repair–a new therapeutic concept? *N Engl J Med* 349:570–582.
6. Chien KR, Moretti A, Laugwitz KL (2004) Development. ES cells to the rescue. *Science* 306:239–240.
7. Nishikawa S, Goldstein RA, Nierras CR (2008) The promise of human induced pluripotent stem cells for research and therapy. *Nat Rev* Mol Cell Biol 9:725–729.
8. Copelan EA (2006) Hematopoietic stem-cell transplantation. *N Engl J Med* 354:1813–1826.
9. Lindvall O, Kokaia Z (2006) Stem cells for the treatment of neurological disorders. *Nature* 441:1094–1096.
10. Segers VF, Lee RT (2008) Stem-cell therapy for cardiac disease. *Nature* 451:937–942.
11. Wang X, et al. (2003) The origin and liver repopulating capacity of murine oval cells. *Proc Natl Acad Sci USA* 100:11881–11888.
12. Griffith LG, Naughton G (2002) Tissue engineering–current challenges and expanding opportunities. *Science* 295:1009–1014.
13. Ikeda E, Tsuji T (2008) Growing bioengineered teeth from single cells: Potential for dental regenerative medicine. *Expert Opin Biol Ther* 8:735–744.
14. Purnell B (2008) New release: The complete guide to organ repair. Introduction. *Science* 322:1489.
15. Lechler RI, Sykes M, Thomson AW, Turka LA (2005) Organ transplantation–how much of the promise has been realized? *Nat Med* 11:605–613.
16. Layer PG, Robitzki A, Rothermel A, Willbold E (2002) Of layers and spheres: The reaggregate approach in tissue engineering. *Trends Neurosci* 25:131–134.
17. Yang J, et al. (2007) Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 28:5033–5043.
18. Nishida K, et al. (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* 351:1187–1196.
19. Miyahara Y, et al. (2006) Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* 12:459–465.
20. Ohashi K, et al. (2007) Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* 13:880–885.
21. Sharpe PT, Young CS (2005) Test-tube teeth. *Sci Am* 293:34–41.
22. Duailibi SE, Duailibi MT, Vacanti JP, Yelick PC (2006) Prospects for tooth regeneration. *Periodontol 2000* 41:177–187.
23. Kuure S, Vuolteenaho R, Vainio S (2000) Kidney morphogenesis: Cellular and molecular regulation. *Mech Dev* 92:31–45.
24. Hogan BL, Yingling JM (1998) Epithelial/mesenchymal interactions and branching morphogenesis of the lung. *Curr Opin Genet Dev* 8:481–486.
25. Pispa J, Thesleff I (2003) Mechanisms of ectodermal organogenesis. *Dev Biol* 262:195–205.
26. Claudinot S, Nicolas M, Oshima H, Rochat A, Barrandon Y (2005) Long-term renewal of hair follicles from clonogenic multipotent stem cells. *Proc Natl Acad Sci USA* 102:14677–14682.
27. Shackleton M, et al. (2006) Generation of a functional mammary gland from a single stem cell. *Nature* 439:84–88.
28. Tucker A, Sharpe P (2004) The cutting-edge of mammalian development; how the embryo makes teeth. *Nat Rev Genet* 5:499–508.
29. Nakao K, et al. (2007) The development of a bioengineered organ germ method. *Nat Methods* 4:227–230.
30. Klingsberg J, Butcher EO (1960) Comparative histology of age changes in oral tissues of rat, hamster, and monkey. *J Dent Res* 39:158–169.
31. Wise GE, King GJ (2008) Mechanisms of tooth eruption and orthodontic tooth movement. *J Dent Res* 87:414–434.
32. Manly RS, Braley LC (1950) Masticatory performance and efficiency. *J Dent Res* 29:448–462.
33. Shimono M, et al. (2003) Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech* 60:491–502.
34. Byers MR, Narhi MV (1999) Dental injury models: Experimental tools for understanding neuroinflammatory interactions and polymodal nociceptor functions. *Crit Rev Oral Biol Med* 10:4–39.
35. Deguchi T, Takeshita N, Balam TA, Fujiyoshi Y, Takano-Yamamoto T (2003) Galanin-immunoreactive nerve fibers in the periodontal ligament during experimental tooth movement. *J Dent Res* 82:677–681.
36. Yen AH, Sharpe PT (2006) Regeneration of teeth using stem cell-based tissue engineering. *Expert Opin Biol Ther* 6:9–16.
37. Ohazama A, Modino SA, Miletich I, Sharpe PT (2004) Stem-cell-based tissue engineering of murine teeth. *J Dent Res* 83:518–522.
38. Song Y, Yan M, Muneoka K, Chen Y (2008) Mouse embryonic diastema region is an ideal site for the development of ectopically transplanted tooth germ. *Dev Dyn* 237:411–416.
39. Duailibi SE, et al. (2008) Bioengineered dental tissues grown in the rat jaw. *J Dent Res* 87:745–750.
40. Zhang W, et al. (2009) Tissue engineered hybrid tooth-bone constructs. *Methods* 47:122–128.
41. Abukawa H, et al. (2009) Reconstructing mandibular defects using autologous tissue-engineered hybrid tooth and bone constructs. *J Oral Maxillofac Surg* 67:335–347.
42. Duailibi MT, et al. (2004) Bioengineered teeth from cultured rat tooth bud cells. *J Dent Res* 83:523–528.
43. Carollo DA, Hoffman RL, Brodie AG (1971) Histology and function of the dental gubernacular cord. *Angle Orthod* 41:300–307.
44. Voruganti K (2008) Clinical periodontology and implant dentistry, 5th edition. *Br Dent J* 205:216.
45. Guyton AC (1967) Textbook of Medical Physiology. *Am J Med Sci* 253:126.
46. Luukko K, Kvinnsland IH, Kettunen P (2005) Tissue interactions in the regulation of axon pathfinding during tooth morphogenesis. *Dev Dyn* 234:482–488.
47. Bengel FM, et al. (2001) Effect of sympathetic reinnervation on cardiac performance after heart transplantation. *N Engl J Med* 345:731–738.
48. Hammerle CH, et al. (1995) Threshold of tactile sensitivity perceived with dental endosseous implants and natural teeth. *Clin Oral Implants Res* 6:83–90.

Regulations of size and shape of the bioengineered tooth by a cell manipulation method

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1. ABSTRACT

Regulation of sizes and shapes of tooth are important matters to consider in generating an entirely bioengineered tooth for future tooth replacement therapy. In our current study, we investigated that whether an extent of contact area between epithelial and mesenchymal cell aggregates, which was reconstituted from embryonic day 14.5 molar tooth germ-derived single cells by our cell manipulation method, affect the morphology of the bioengineered tooth. Statistical analysis showed that there were reliable correlations between the contact length of bioengineered tooth germ and the crown widths (R=0.84), and the cusp numbers (R=0.85). These observations indicate that the crown widths and the cusp numbers of bioengineered molar were determined by the contact length between epithelial and mesenchymal cell layers. Our study results will provide important insights into the regulatory mechanisms of sizes and shapes of bioengineered tooth and the application of cell manipulation technology in future tooth replacement therapy.

2. INTRODUCTION

The current approaches being used to develop future regenerative therapies are influenced by our understanding of embryonic development, stem cell biology, and tissue engineering technology [1-4]. One of the more attractive concepts under consideration in regenerative therapy is stem cell transplantation of enriched or purified tissue-derived stem cells [5], or in vitro manipulated embryonic stem (ES) and induced pluripotent stem (iPS) cells [6, 7]. This therapy has the potential to restore the partial loss of organ function by replacing hematopoietic stem cells in hematopoietic malignancies [8], neural stem cells in Parkinson's disease [9], mesenchymal stem cells in myocardial infarction [10], and hepatic stem cells in cases of hepatic insufficiency [11].

The ultimate goal of regenerative therapy is to develop fully functioning bioengineered organs that can replace lost or damaged organs following disease, injury, or aging [4, 12-14]. The feasibility of this concept has essentially been demonstrated by successful organ transplantations for various injuries and diseases [15]. It is expected that bioengineering technology will be developed for the reconstruction of fully functional organs in vitro through the precise arrangement of several different cell species [3, 16-20]. We have recently reported a successful fully functioning tooth replacement in an adult mouse achieved through the transplantation of bioengineered tooth germ into the alveolar bone in the lost tooth region. We propose this technology as a model for future organ replacement therapies. The bioengineered tooth, which was erupted and occluded, had the correct tooth structure, hardness of mineralized tissues for mastication, and response to noxious stimulations such as mechanical stress and pain in cooperation with other oral and maxillofacial tissues [21, 22]. However, the bioengineered tooth was smaller than the other normal teeth, since at present we cannot regulate the crown width, cusp position, and tooth patterning including anterior/posterior and buccal/lingual structures using in vitro cell manipulation techniques.

Here, we showed that the crown widths and the cusp numbers of bioengineered molar could be regulated by cell manipulation method, and were determined by the contact length between epithelial and mesenchymal cell layers but not dependent on the cell number.

3. MATERIALS AND METHODS

3.1 Animals

C57BL/6 mice were purchased from SLC Inc. Mouse care and handling conformed to the NIH guidelines for animal research. All experimental protocols were approved by the Tokyo University of Science Animal Care and Use Committee.

3.2 Reconstitution of bioengineered tooth germ from single cells

Molar tooth germs were dissected from the mandibles of ED14.5 mice. The isolation of tissues and each single cell preparation from epithelium and mesenchyme has been described previously. Dissociated epithelial and mesenchymal cells were precipitated by centrifugation in a siliconized microtube and the supernatant was completely removed. The cell density of the precipitated epithelial and mesenchymal cells after the removal of supernatants reached

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a concentration of 5 x 10^8 cells/mL as described previously [21]. Bioengineered molar tooth germ was reconstituted using our previously described 3-dimensional cell manipulation method, the 'organ germ method' [21]. We reconstituted various bioengineered tooth germs, which have various contact length between epithelial and mesenchymal cell layers, with uniform thickness using a micro-syringe of 0.330 mm inner diameter (Hamilton). These various bioengineered tooth germs were incubated for 10 min at 37 °C, placed on a cell culture insert (0.4 μm pore diameter; BD), and then further incubated at 37 °C for 5 days in an in vitro organ culture as described previously [21].

3.3 SRC Assay

After 7 dayss incubation, the reconstituted germs of tooth or whisker follicles were transplanted into a subrenal capsule (SRC) for 21 days using 8 week-old male mice as the host, according to the method of Bogden and co-workers [23].

3.4 Microcomputed Tomography (MicroCT)

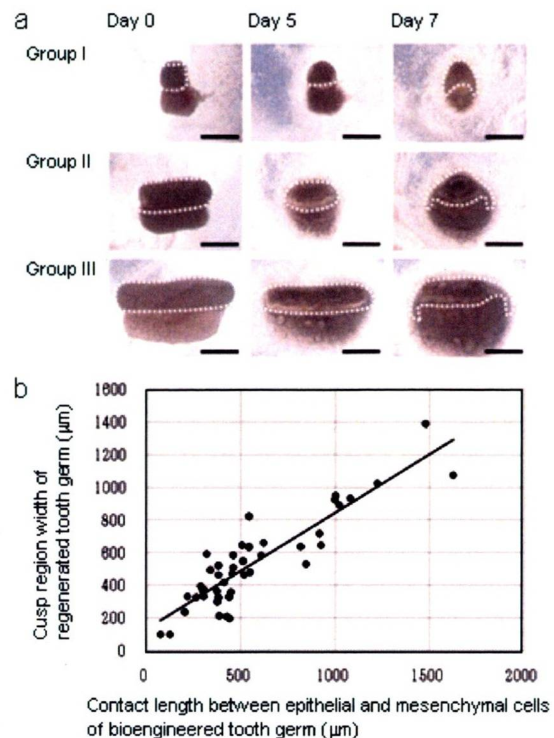
The heads of mice implanted with bioengineered tooth germ and control mice were fixed in the centric occlusal position and radiographic imaging was then performed by x-ray using an inspeXio SMX-90CT device (Shimadzu) with exposure at 90 kV and 0.1 mA, and with a source-to-sample distance of 34 mm. Microcomputed tomography was performed using Imaris (Carl Zeiss).

3.5 Tissue Preparation

The tissues were removed and immersed in 4% paraformaldehyde in PBS(-). After fixation, the tissues were decalcified in 4.5% EDTA (pH 7.4) for 1-10 days at 4°C. The sections were observed using an Axio Imager A1 (Carl Zeiss, Jena, Germany) with an AxioCAM MRc5 (Zeiss) and processed with AxioVision software (Zeiss).

4. RESULTS AND DISCUSSION

We first investigated that whether an extent of contact area between epithelial and mesenchymal cell aggregates, which was reconstituted from embryonic day 14.5 molar tooth germ-derived single cells by our cell manipulation method, affect the morphology of the bioengineered tooth. We reconstituted various bioengineered tooth germs, which have various contact length between epithelial and mesenchymal cell layers, with uniform thickness using a micro-syringe of 0.330 mm inner diameter. After a day of organ culture, we categorized into three-types of the bioengineered tooth germ by the contact length: group I, up to 450 μm; group II, 450-900 μm; and group III, 900-1500 μm. The bioengineered molar tooth germ in group I to III had developed at the early bell stage of a natural tooth germ after 5-7 days growth and were with a mean width of each 366 ± 103 μm, 584 ± 103 μm and 934 ± 239 μm, respectively (Fig. 1a).



F
Regulation of the width of bioengineered tooth germ by cell manipulation method. (a) Phase contrast image of three-types of the bioengineered tooth germ on day 0, day 5 and day 7 of an organ culture. (Scale bar, 500 μm.) (b) Scatter diagram of correlational analysis between the contact length and the width of the bioengineered tooth germ.

Statistical analysis revealed that there were significant correlations between the contact length and the mean width of the bioengineered molar tooth germs (R=0.89) (Fig. 1b). After 21 days transplantation in subrenal capsule, the bioengineered molars developed from the bioengineered germs in group I to III were with a mean crown widths of each 497 ± 118 μm, 727 ± 271 μm and 1073 ± 186 μm, respectively (Fig. 2a). The bioengineered tooth formed a correct structure comprising enamel, ameloblast, dentin, odontoblast, dental pulp, alveolar bone, and blood vessels (Fig. 2a). Moreover, the cusp numbers of those teeth derived from the bioengineered germs in group I to III were 2.9 ± 0.8, 4.7 ± 3.1 and 11 ± 2.6, respectively (Fig. 2a). Statistical analysis showed that there were reliable correlations between the contact length of bioengineered tooth germ and the crown widths (R=0.84) (Fig. 2b), and the cusp numbers (R=0.85) (Fig. 2c).
Finally, we examined that whether the cell number of epithelial and mesenchymal cells influences the morphology of the bioengineered tooth. We formed the cell aggregates with different thickness by use of micro-syringe of 0.203

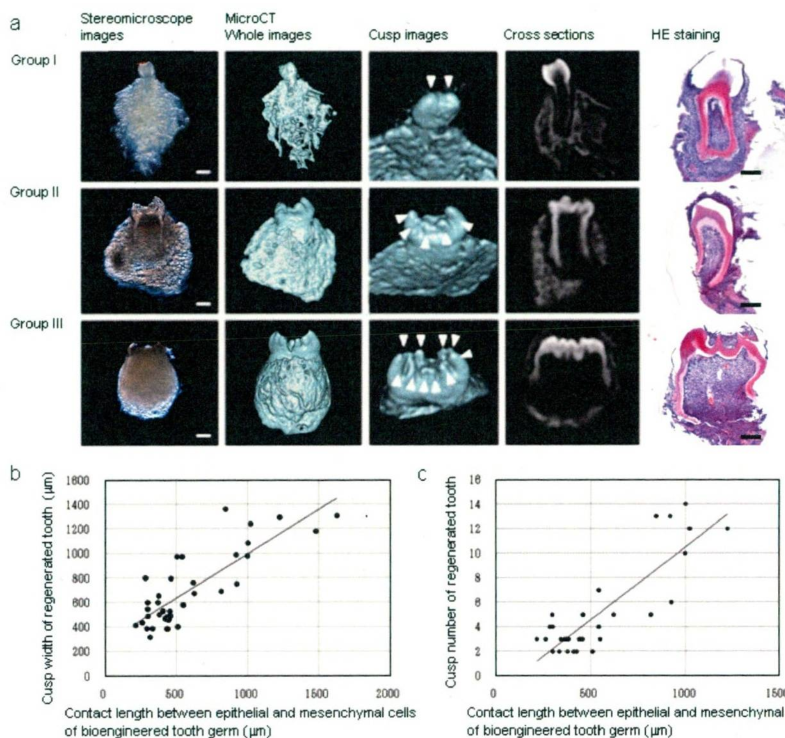


Fig. 2
Regulation of the crown width and cusp number of bioengineered tooth by cell manipulation method. (a) Morphological and histological analysis of three-types of the bioengineered tooth after 21 days transplantation. Stereomicroscope images (first columns from the left), whole images of MicroCT (second columns), Cusp images (third columns), cross sections (fourth columns) are shown. Arrow head, cusp. (Scale bar, 200 μm). (b) Scatter diagram of correlational analysis between the contact length and the crown width of the bioengineered tooth. (c) Scatter diagram of correlational analysis between the contact length and the cusp number of the bioengineered tooth.

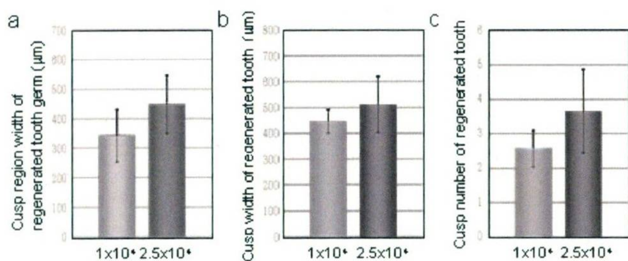


Fig. 3
Morphological differences of the bioengineered tooth by regulating cell number. Cusp region width of the bioengineered tooth germ on day 7 organ culture (a), crown width (b) and cusp number (c) of the bioengineered tooth after 21 days transplantation are shown.

mm or 0.330 mm inner diameter while limiting the contact length to 300-500 μm. However, there were no differences in the crown widths and cusp numbers of the bioengineered molar between $1x10^4$ cells per aggregate and $2.5x10^4$ cells per aggregate ($p>0.06$) (Fig. 3a, b and c). These results indicated that the crown widths and the cusp numbers of bioengineered molar were determined by the contact length between epithelial and mesenchymal cell layers but not dependent on the cell number.

In conclusion, this study provides an important insight into the regulatory mechanisms of sizes and shapes of bioengineered tooth and the application of cell manipulation technology in future tooth replacement therapy. Further studies on the identification of available adult tissue stem cells for the reconstitution of a bioengineered tooth germ and the regulation of stem cell differentiation into odontogenic cell lineage will help to achieve the realization of tooth regenerative therapy for missing teeth.

5. ACKNOWLEDGEMENT

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6. REFERENCES

1. Brockes JP, Kumar A. *Science* **310**, 1919–1923 (2005).
2. Watt FM, Hogan BL. *Science* **287**, 1427–1430 (2000).
3. Langer RS, Vacanti JP. *SciAm***280**, 86–89 (1999).
4. Atala A. *Expert Opin Biol Ther* **5**, 879–892 (2005) .
5. Korbling M, Estrov Z. *N Engl J Med* **349**, 570–582 (2003).
6. Chien KR, Moretti A, Laugwitz KL. *Science* **306**, 239–240 (2004).
7. Nishikawa S, Goldstein RA, Nierras CR. *Nat Rev* Mol Cell Biol **9**, 725–729 (2008).
8. Copelan EA. *N Engl J Med* **354**, 1813–1826 (2006).
9. Lindvall O, Kokaia Z. *Nature* **441**, 1094–1096 (2006).

10. Segers VF, Lee RT. *Nature* **451**, 937–942 (2008).
11. Wang X, et al. *Proc Natl Acad Sci USA* **100**, 11881–11888 (2003).
12. Griffith LG, Naughton G. *Science* **295**, 1009–1014 (2002).
13. Ikeda E, Tsuji T. *Expert Opin Biol Ther* **8**, 735–744 (2008).
14. Purnell B. *Science* **322**, 1489 (2008).
15. Lechler RI, Sykes M, Thomson AW, Turka LA. *Nat Med* **11**, 605–613 (2005).
16. Layer PG, Robitzki A, Rothermel A, Willbold E. *Trends Neurosci* **25**, 131–134 (2002).
17. Yang J, et al. *Biomaterials* **28**, 5033–5043 (2007).
18. Nishida K, et al. *N Engl J Med* **351**, 1187–1196 (2004).
19. Miyahara Y, et al. *Nat Med* **12**, 459–465 (2006).
20. Ohashi K, et al. *Nat Med* **13**, 880–885 (2007).
21. Nakao K, et al. *Nat Methods* **4**, 227–230 (2007).
22. Ikeda E, Morita R, et al. *Proc Natl Acad Sci USA,* Epub ahead of print (2009).

Strategies underlying research in tooth regenerative therapy as a possible model for future organ replacement

Kazuhisa Nakao and Takashi Tsuji

Abstract. The ultimate goal of regenerative therapy is to develop fully functioning bioengineered organs that can replace lost or damaged organs after disease, injury, or aging. We have previously developed a three-dimensional culture system with the aim of reconstituting a bioengineered organ germ at an early developmental stage. The regeneration of a functional tooth unit is critical issue to achieving proper oral function, including mastication. Recently, we successfully demonstrated that our bioengineered tooth germ could develop a fully functioning tooth with sufficient hardness for masticatory potential, the ability to withstand mechanical stress in the maxillofacial region, and in which the innerved neural fibers had an adequate perceptive potential for noxious stimulations. Our results thus show that bioengineered tooth germ can develop a fully functioning regenerated tooth in vivo after engraftment and therefore that organ replacement regenerative therapy in this way is feasible.

Key words. regenerative therapy, tooth, organ germ method, bioengineered organ, transplantation

1 Introduction

Regenerative medicine is an anticipated clinical application in coming years [1–4], and stem cell transfer therapy (in which stem cells are removed and transferred to damaged organs and tissues of the same individual) has already begun to be developed [5]. The ultimate goal of regenerative medicine is to replace a defective organ with artificially regenerated organ that has full functionality [6–9]. In the dental field, therapies have

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been established to replace teeth, which are ectodermal organs, with artificial implants that can provide some functions [10–12]. However, although these treatments can effectively substitute a lost tooth, transplantations of a natural tooth such as third molar are being attempted to provide more biological functionality [13]. For next-generation therapies, it is expected that tooth replacement will involve transplantation of a bioengineered tooth which has been constructed from stem cells [7,10,11].

2 The Strategies Underlying Current Research on Tooth Regenerative Therapy

In current research on whole-tooth regenerative therapies, one of the basic underlying strategies involves the transplantation of bioengineered tooth germ, which can then develop into a fully functional tooth [7,10,11]. Teeth arise from the tooth germ, which is induced by reciprocal epithelial-mesenchymal interactions in the developing embryo [14,15]. The epithelium and the mesenchyme differentiate into ameloblasts, which later become enamel and odontoblasts, respectively, which will form dentin. The mesenchyme also differentiates into dental pulp and into periodontal tissues, which will become cementum, alveolar bone, and periodontal ligament (PDL).

As described by many experts in the dental field, four major hurdles need to be overcome to enable the development of tooth regenerative therapy [7,10,11]. The first is the establishment of a more effective bioengineering method of producing three-dimensional organ germs from single cells. The second is the development of this bioengineered tooth germ in an adult oral environment. The third relates to how dental regenerative therapy may be optimized using the patient's own cells. Finally, the transplantation of a morphologically controlled regenerated tooth would be improved by more effective in vitro organ processing.

3 The Development of a Novel Bioengineered Organ Germ Method

Previously in our laboratory, we investigated the feasibility of developing a bioengineering cell processing method for three-dimensional organ germ using single cells. To precisely replicate the process of tooth organogenesis at early developmental stages, we employed a cell aggregation method using epithelial cells and mesenchymal cells isolated from cap stage tooth germ from the lower jaw of E14.5 mice. The epithelial and mesenchymal single cells were prepared using enzymatic treatments (Fig. 1a). Explants that reconstituted the cell compartmentalization between epithelial and mesenchymal cells at a low-cell density ($0.5–1 \times 10^8$ cells/ml), or that did not form cell compartmentalization at high-cell density (5×10^8 cells/ml), failed to generate a correct tooth structure. To reconstitute a bioengineered tooth germ with the correct cell compartmentalization between epithelial and mesenchymal-derived single cells, these cells were injected in turn at a high density (5×10^8 cells/ml) into

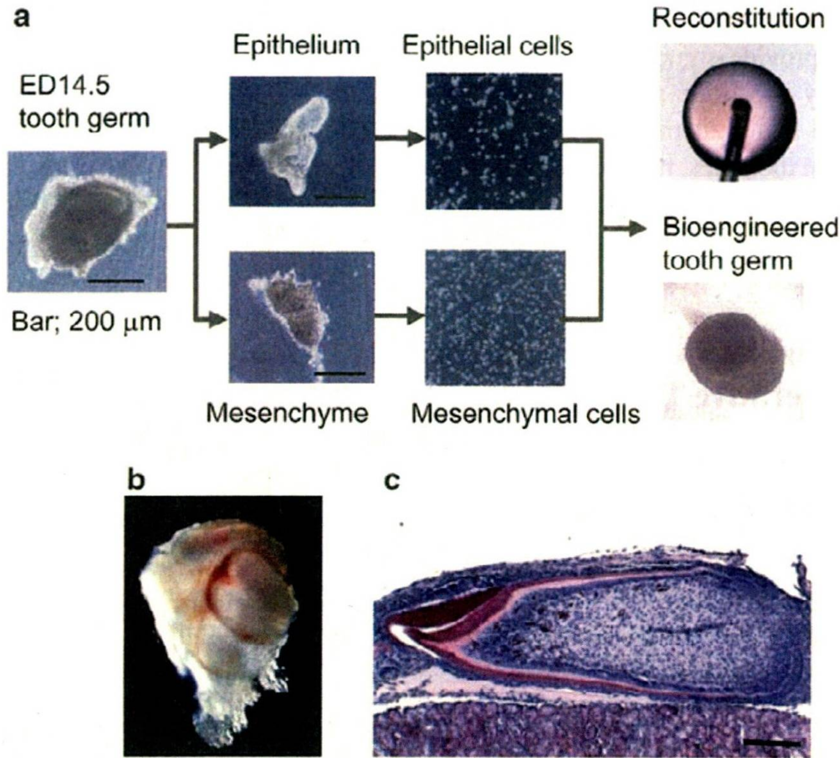

Fig. 1. Generation of a whole tooth using bioengineered tooth germ derived from dissociated single cells. (**a**) Schematic of the bioengineering technology used for the generation of a reconstituted tooth germ. (**b**) Representative phase contrast images showing a bioengineered tooth developed in a subrenal capsule environment for 14 days. (**c**) Histological analysis of the reorganized tooth germ under a subrenal capsule for 14 days. Scale bar, 250 μm

a collagen gel drop (Fig. 1a). Within 2 days of organ culture, a tooth germ was observed to form with the appropriate compartmentalization and cell–cell compaction. At 14 days after the transplantation into a subrenal capsule, this germ could successfully generate plural teeth in the alveolar bone of the mouse (Fig. 1b, c). These results emphasize that a high-cell density and a correct cell compartmentalization are essential for a bioengineered tooth to develop properly, and we termed our successful approach in this regard as a "bioengineered organ germ method" [16]. Our model improves the current understanding of the principles by which organ reconstitution can be achieved with tissues that have been bioengineered in vitro and increases the potential for bioengineered organ replacement in the future.

4 Eruption and Structure af a Bioengineered Tooth

We further investigated whether bioengineered molar tooth germ reconstituted using our novel method could erupt in an adult murine lost tooth transplantation model [17]. We extracted the first molar from the subject mice and allowed the cavity to

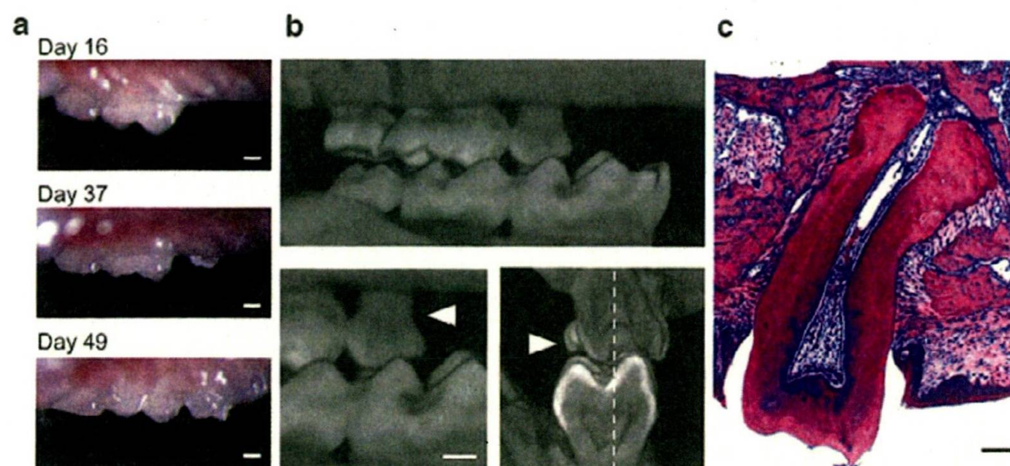

Fig. 2. Eruption and occlusion of a bioengineered tooth. (**a**) Oral photographs of the bioengineered tooth during eruption and occlusion processes. Scale bar, 200 μm. (**b**) MicroCT image of the occlusion of a bioengineered tooth (*arrowhead*). (**c**) Histological analysis of the bioengineered tooth in full occlusion. Scale bar, 100 μm

undergo repair by osteogenesis for 1 month. We then drilled a hole in the alveolar bone and transplanted our bioengineered molar tooth germ into this cavity in the correct orientation. At 16 days after this transplantation, eruption of the bioengineered tooth could not be observed (Fig. 2a). However, at 37 days post-transplantation, a cusp tip could be observed in the gingival area of the transplantation site, indicating an eruption of the bioengineered tooth. At 49 days post-transplantation and thereafter, this bioengineered tooth was observed to reach the occlusal plane and achieve opposing tooth occlusion (Fig. 2a, b). Following the achievement of occlusion, there was no excessive increase found in the tooth length at 120 days post-transplantation [17]. The bioengineered tooth also formed a correct structure comprising enamel, ameloblasts, dentin, odontoblasts, dental pulp, alveolar bone, and blood vessels (Fig. 2c). These results indicated that the tooth tissue structures of the bioengineered tooth were similar to those of a normal tooth [17].

5 Functional Bioengineered Tooth Replacement In an Adult Oral Environment

To develop a tooth regenerative method for possible future clinical applications, a bioengineered tooth must necessarily achieve full functionality, including sufficient masticatory performance [18], biomechanical cooperation with tissues in the oral and maxillofacial regions [19], and proper responsiveness via sensory receptors to noxious stimulations in the maxillofacial region [20]. Masticatory potential in particular is essential for achieving proper tooth function [18]. Significantly, the hardness of the mineralized tissues within our bioengineered tooth, as analyzed by

the Knoop hardness test and including both enamel and dentin, was equivalent to that of a normal tooth. These results indicated an equivalent masticatory performance to a normal, mature tooth [17].

It has been established previously that alveolar bone remodeling is induced by the response of the PDL to mechanical stress such as orthodontic movement [19]. We found in this regard that our regenerated tooth achieved normal occlusion in harmony with other teeth in the recipient mouse and also displayed opposing cuspal contacts that maintained a proper occlusal vertical dimension between the opposing arches (Fig. 2b). The bioengineered tooth could also successfully move in response to mechanical stress as well as a normal tooth [17]. These findings indicate that the PDL of the bioengineered tooth successfully mediate bone remodeling in response to mechanical stress induced by the experimental orthodontic treatment.

The perception of noxious stimulations such as mechanical stress and pain is an important tooth function [20]. We found that the nerve fibers innervating both the pulp and the PDL of our bioengineered tooth had perceptive potential for nociceptive stimulation (Fig. 3) and could transduce these events to the central nervous system (the medullary dorsal horn) [17]. In these analyses, we observed that our bioengineered tooth germ developed into a fully functioning tooth with sufficient hardness for mastication and a functional responsiveness to mechanical stress in the maxil-

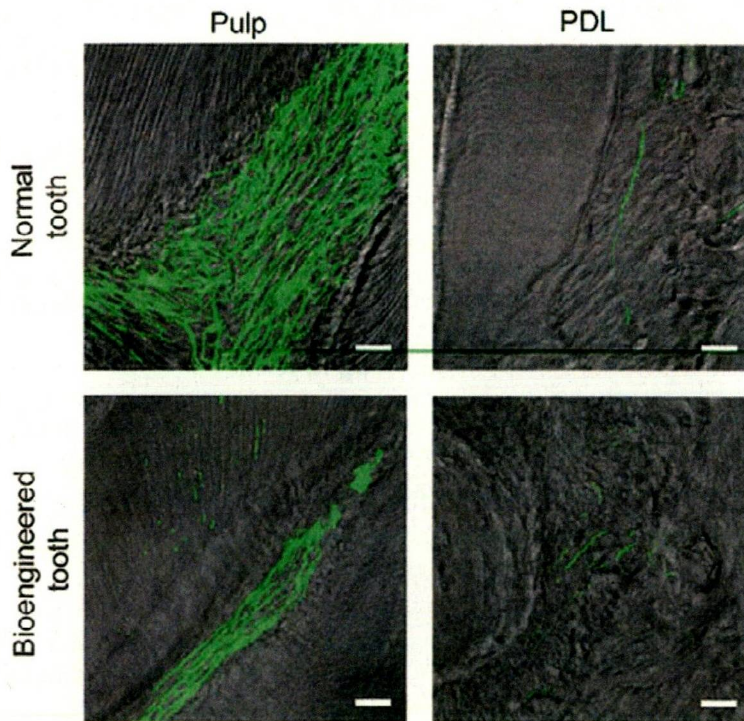

Fig. 3. Immunohistochemical analysis of nerve fibers entering the tissue of the bioengineered tooth. Nerve fibers in the pulp and periodontal ligament (PDL) in the normal and the bioengineered tooth were analyzed immunohistochemically using specific antibodies for neurofilament-H. Scale bar, 25 μm

lofacial region. We further confirmed that the neural fibers that has re-entered the pulp and PDL tissues of the bioengineered tooth had a proper level of perceptive potential in response to noxious stimulations such as orthodontic treatment and pulp stimulation. These findings further indicate that the bioengineered tooth generation techniques we developed can contribute to the rebuilding of a fully functional tooth.

6 Conclusions

We have provided the first description of a successful replacement of an entire and fully functioning organ in an adult body through the transplantation of bioengineered organ germ reconstituted by single cell manipulation in vitro. Our studies have therefore made a substantial contribution to the future development of bioengineering technology for organ replacement therapy. Further studies on the identification of available adult tissue stem cells for the reconstitution of a bioengineered tooth germ and the regulation of tooth shape will help to achieve the realization of tooth regenerative therapy in humans.

References

1. Brockes JP, Kumar A (2005) Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310:1919–1923
2. Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. Science 287:1427–1430
3. Langer RS, Vacanti JP (1999) Tissue engineering: the challenges ahead. Sci Am 280:86–89
4. Atala A (2005) Tissue engineering, stem cells and cloning: current concepts and changing trends. Expert Opin Biol Ther 5:879–892
5. Korbling M, Estrov Z (2003) Adult stem cells for tissue repair – a new therapeutic concept? N Engl J Med 349:570–582
6. Griffith LG, Naughton G (2002) Tissue engineering – current challenges and expanding opportunities. Science 295:1009–1014
7. Ikeda E, Tsuji T (2008) Growing bioengineered teeth from single cells: potential for dental regenerative medicine. Expert Opin Biol Ther 8:735–744
8. Purnell B (2008) New release: the complete guide to organ repair. Introduction. Science 322:1489
9. Lechler RI, Sykes M, Thomson AW et al (2005) Organ transplantation – how much of the promise has been realized? Nat Med 11:605–613
10. Sharpe PT, Young CS (2005) Test-tube teeth. Sci Am 293:34–41
11. Duailibi SE, Duailibi MT, Vacanti JP et al (2006) Prospects for tooth regeneration. Periodontol 2000 41:177–187
12. Voruganti K (2008) Clinical periodontology and implant dentistry, 5th edition. Br Dent J 205:216
13. Tsukiboshi M (1993) Autogenous tooth transplantation: a reevaluation. Int J Periodontics Restorative Dent 13(2):120–149
14. Pispa J, Thesleff I (2003) Mechanisms of ectodermal organogenesis. Dev Biol 262:195–205
15. Tucker A, Sharpe P (2004) The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet 5:499–508

16. Nakao K, Morita R, Saji Y et al (2007) The development of a bioengineered organ germ method. Nat Methods 4:227–230
17. Ikeda E, Morita R, Nakao K et al (2009) Fully functional bioengineered tooth replacement as an organ replacement therapy. Proc Natl Acad Sci U S A 106:13475–13480
18. Manly RS, Braley LC (1950) Masticatory performance and efficiency. J Dent Res 29:448–462
19. Shimono M, Ishikawa T, Ishikawa H et al (2003) Regulatory mechanisms of periodontal regeneration. Microsc Res Tech 60:491–502
20. Byers MR, Narhi MV (1999) Dental injury models: experimental tools for understanding neuroinflammatory interactions and polymodal nociceptor functions. Crit Rev Oral Biol Med 10:4–39

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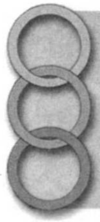

Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology

ORAL MEDICINE *Editor: Craig S. Miller*

The relationship between temporomandibular joint pathosis and muscle tenderness in the orofacial and neck/shoulder region

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Objective. The objective of this study was to investigate the association between TMJ pain/disk pathosis and the muscle tenderness pattern in the orofacial and neck/shoulder region.

Study design. One hundred seventy-one TMD patients were divided into 4 groups, including group 1: patients with painful unilateral TMJ disk displacement (DD); group 2: patients with painless unilateral TMJ DD; group 3: patients with painless bilateral TMJ DD; and group 4: patients with a bilateral normal TMJ disk position (n = 41). Each subject underwent muscle palpation and the side-by-side number of muscle tenderness points was combined as the number of muscle tenderness points on each side. Within each group, DD with and without reduction subjects were separated into subgroups and then were analyzed.

Results. In group 1, the median muscle tenderness points on the side with painful TMJ DD without reduction was significantly higher than on the normal side (P = .019), whereas the palpation scores for painless DD patients showed no significant difference between the DD and normal sides.

Conclusions. These results indicated painful disk displacement to possibly be correlated with ipsilateral muscle tenderness. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:86-90)**

The main signs and symptoms of temporomandibular disorders (TMD) of articular origin are joint pain, joint noise, and limitations in opening the mouth. In addition, other various symptoms may occur, including masticatory and other orofacial muscle pain, headache, shoulder stiffness, earache, and tinnitus.[1-5] Temporomandibular joint (TMJ) pain refers to muscles in the orofacial region. Furthermore, primary pain in one of the structures may also lead to secondary changes in another site that then becomes a further source of pain and functional impairment.[6-8] However, the clinical or basic research findings are not sufficient to unequivocally state that TMJ and muscle symptoms are closely related or that they comorbidly arise from 2 different conditions. Previously, Seligman et al.[9] demonstrated that subjects with muscle tenderness tended to have more TMJ clicking than those without muscle tenderness. Wanman[10] investigated the relationship between muscle tenderness and signs and symptoms of TMD (such as TMJ noise, pain, tenderness, and locking) using a random sample of 345 subjects 35 years of age. Wanman found the presence of a signifi-

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cantly higher proportion of signs and symptoms of TMD in the group who had both jaw muscle tenderness and neck/shoulder muscle tenderness. Symptom-based analyses between TMJ and masticatory, neck/shoulder muscles have been performed by several authors; however, no study has investigated the direct relationship between TMJ pathosis and pain in the muscles surrounding the TMJ. The abnormal TMJ disk position is closely associated with several TMD signs and symptoms (e.g., joint noise, limitation of mouth opening). Therefore, it is considered to be clinically worthwhile to analyze the relationship between TMJ disk position abnormalities diagnosed by MRI and muscle pain patterns. Hopefully such knowledge will be useful for diagnosis and predicting the prognosis of TMD. Therefore this study investigated the association between the TMJ disk position, diagnosed by magnetic resonance imaging (MRI) and muscle pain in the orofacial and neck/shoulder region by comparing the prevalence of muscle tenderness in both disk displacement and contralateral normal disk position sides. In addition, the effect of pain in the TMJ on the tenderness in ipsilateral muscle tissues was also evaluated using same patient groups.

MATERIALS AND METHODS

Study population

The subjects were selected from a series of TMD patients (453 patients, 38.7 ± 19.1 years, male/female = 120/333) who attended a TMD clinic in Department of Fixed Prosthodontics in Okayama University Dental Hospital from May 1997 to May 2002. The eligible subjects were selected (219 patients, 37.6 ± 18.7 years, male/female = 59/160) according to the following inclusion criteria: patients who were diagnosed with a TMJ condition by clinical examination and MRI according to the operational criterion (IZ criterion) described by Orsini et al.[11] This approach involves the description of the location of the intermediate zone (IZ) of the disk in relation to the condyle and the articular eminence. The thin central portion of disk (IZ) was identified; when IZ was located between the anterior-superior aspect of the condyle and the posterior-inferior aspect of the articular eminence in the middle or above a line that joined centers of 2 imaginary circles fitted to these structures, the position of the disk was considered normal. The disk position during opening was considered normal if IZ of the disk was located between the condyle and articular eminence (when the jaw was wide open) in the middle of a line that joined centers of 2 imaginary circles fitted to these structures.

Subjects were excluded if they presented with one or more of the following conditions: (1) a history of rheumatoid arthritis, systemic osteoarthritis, and systemic lupus erythematosus; (2) patients who had pain in the unilateral TMJ even though both TMJ disks were displaced; (3) patients who had bilateral painful TMJ disk displacement, because of the small number of such patients in the sample a statistical analysis could not be performed (10 subjects: 5 had painful disk displacement with reduction whereas the other 5 had painful disk displacement without reduction). A total of 48 patients were excluded by not filling in their questionnaires on their TMD signs and symptoms, so that final sample included 171 patients (35.3 ± 18.0 years, male/female = 47/124). The patients were then divided into the following 4 experimental groups: group 1, unilateral painful TMJ disk displacement (n = 58); group 2, unilateral painless TMJ disk displacement (n = 51); group 3, bilateral painless TMJ disk displacement (n = 21); group 4, patients whose TMJ disk positions on both sides were normal (n = 41). Within each group, disk displacement with and without reduction subjects were separated into subgroups and then were analyzed.

This study protocol was approved by an appropriate ethics committee in Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (#144).

Muscle palpation

Each patient underwent a clinical digital head and neck muscle palpation at the initial visit by 1 of 3 calibrated examiners (K.M, H.M, and T.K). Each muscle palpation site (Fig. 1) on both sides of the head was palpated using a steady 2 kgf compression force for 3 seconds. The muscle tenderness was assessed using the modified Krogh-Poulsen's method.[12] This method involves 16 palpation sites from 6 muscles (masseter: 5 sites, temporalis: 3 sites, medial pterygoid: 1 site, sternocleidomastoid: 3 sites, digastric: 2 sites, and trapezius: 2 sites); tenderness level in each site was evaluated using 4-graded pain assessment score from 0 to 3 (0: nonpainful, 1: mild, 2: moderate, 3: severe). Thereafter, the tenderness score at each site was just divided into 2 scores (0 or 1) and submitted to statistical analysis. Scores of 1 to 3 were defined as "1" meaning painful and a score of 0 remained nonpainful (score "0"). The total number of muscle tenderness points on the right or left side (maximum: 16) was calculated and these scores were analyzed.

Statistical analysis

The comparisons of demographic data between intended and actual samples were made using the *t* test and the chi-square test. To investigate the relationship between the muscle tenderness pattern and TMJ disk position, the total number of muscle tender points were compared between both joints in each individual using the Wilcoxon rank sum test. The statistics were separately performed in each experimental group. In addition, we analyzed the data to evaluate if any other factors could be the confounders of the findings. Specifically, the relationship between the subjects' age, gender, and the number of muscle tender points were evaluated. The number of

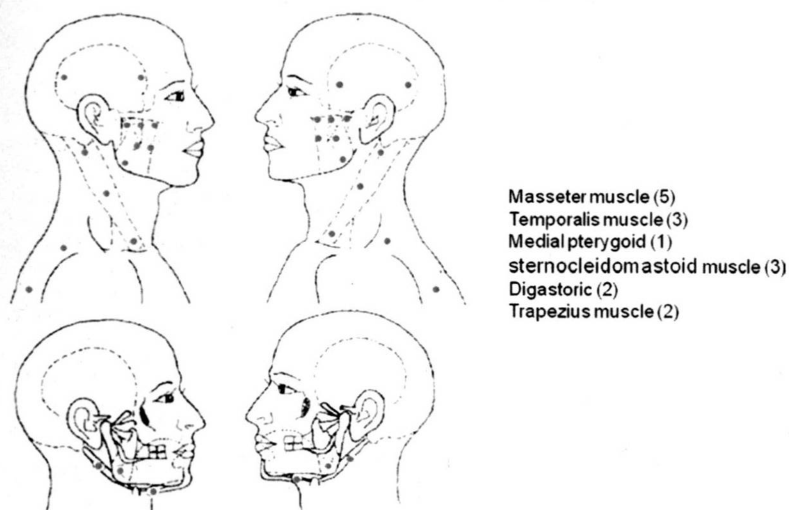

Fig. 1. Orofacial, neck-shoulder muscle palpation sites examined in this study. Gray circles represent the anatomic locations and the numerals in the parentheses indicate the number of the points that were palpated in each muscle.

Table I. Baseline comparisons of demographic data in each subject group

	Mean age			*Gender distribution (Male/Female)*		
	Intended sample	*Actual sample*	*P value*	*Intended sample*	*Actual sample*	*P value*
Group 1	37.3 ± 18.8	36.3 ± 19.1	0.47	14/55	12/46	.96
Group 2	32.3 ± 17.3	29.9 ± 16.4	0.69	17/45	16/35	.65
Group 3	31.8 ± 15.2	29.6 ± 13.0	0.63	2/23	2/19	.86
Group 4	45.2 ± 19.0	43.9 ± 17.9	0.21	26/37	17/24	.98
Total	37.6 ± 18.7	35.3 ± 18.0	0.76	59/160	47/124	.90

Mean age *t-test*.
Gender distribution *Chi-square test*.

muscle tender points between each gender were compared using the Wilcoxon rank sum test. Any correlations between the number of muscle tender points and the subjects' age were determined by Pearson's product *r*-value. A value of *P* less than .05 was considered to be significant.

RESULTS

Baseline comparisons

Table I shows the baseline comparisons of the demographic data in each subject group. These data suggest that the mean age and gender distribution between the intended and actual sample populations did not differ in each subject group. In other words, homogeneity was preserved even after excluding a number of subjects owing to an incomplete response to the questionnaire.

Muscle tenderness

Fig. 2 shows the comparisons of the median number of muscle palpation tender points between the disk displacement (DD) side and control side (right and left side in group 3 and 4). In group 1, the median number of the muscle tender points in the DD without reduction side was significantly higher than that of contralateral normal disk position side ($P = .019$). On the other hand, no statistically significant difference was observed; similar tendency was observed in comparisons between the DD with reduction side and the contralateral normal side in group 1. On the other hand, in the

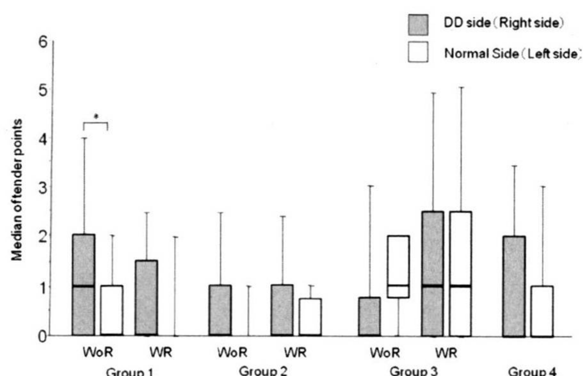

Fig. 2. Comparisons of the median of muscle tender points between the disk displacement (DD) side and the contralateral normal side (right and left side in group 3 and 4). WoR, disk displacement without reduction; WR, disk displacement with reduction. $^{*}P < .05$ (Wilcoxon rank sum test).

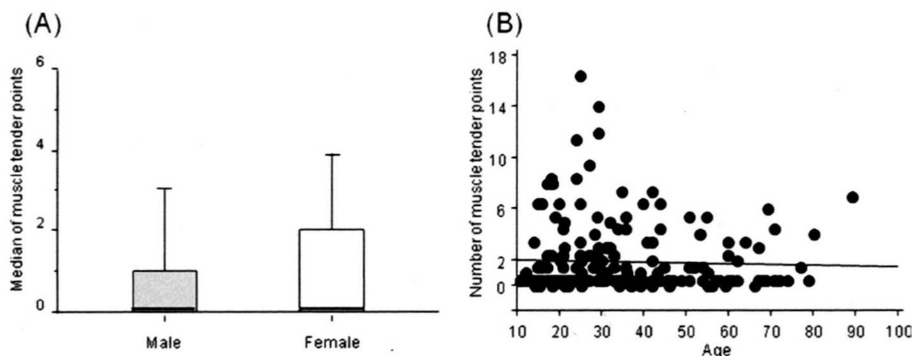

Fig. 3. Comparisons of the median number of muscle tender points between each gender (**A**) and the relationship between the subjects' age and the number of muscle tender points (**B**).

unilateral DD without joint pain patients (group 2), no significant median difference was observed in the muscle palpation tender points between the DD and contralateral normal sides. The median number of the muscle tender points did not differ between the right and left side in groups 3 and 4. These results suggest that muscle tenderness does not differ in a consistent relationship to the disk position between the right and left joints in these patients. Regarding gender difference, the median number of muscle tender points did not differ between each gender (P = .34, Fig. 3, *A*). No significant correlation was observed between the subjects' age and the number of muscle tender points (r = −0.034; P = .65, Fig. 3, *B*).

DISCUSSION

This study investigated the association between TMJ pain/disk pathology and the muscle tenderness pattern in the orofacial region. No clear association was identified between the muscle tenderness side and the TMJ DD side. It was only when the subjects with DD had a concurrent complaint of joint pain that the muscle palpation score was also elevated. These results suggest the possibility that disk displacement itself does not directly induce pain in the ipsilateral muscle tissues. One of the possible mechanisms of the observed phenomena is, as briefly mentioned in the introduction, that either the inflammatory pain or discal attachment tissue impingement pain in the TMJ induces a referred pain in the adjacent muscle tissues. Several clinical studies have proposed this concept,[13-15] and basic animal research findings, which support this hypothesis, have also been reported. Specifically, Kojima[16] investigated the convergence patterns of afferent information from the TMJ and the masseter muscle in the trigeminal subnucleus caudalis in response to natural stimulation in anesthetized rats. He found that afferent inputs from the TMJ and the masseter muscle converged on 108 (80%) of the 135 neurons and of these convergent neurons, 79% received nociceptive information from the TMJ and/or the masseter. Ohya[17] studied the responses of trigeminal subnucleus interpolaris neurons to stimulation of the TMJ and the masseter muscle in a rat model. These results suggested that approximately 70% of the interpolaris neurons receive nociceptive inputs from the TMJ and/or the masseter and most of these neurons have an extensive convergence of afferent inputs, including the TMJ, masseter, and facial skin. Finally, the most recent study reported by Morch et al.[18] demonstrated the convergence of the musculoskeletal (including TMJ and masseter muscle) afferents onto nociceptive neurons in the first cervical dorsal horn. They proposed that the afferent convergence in first cervical dorsal horn nociceptive neurons may be limited to the craniofacial area and that they may play an important role in the integration of craniofacial and upper cervical nociceptive inputs. Therefore, the results obtained in the current study are consistent with the pain referral mechanisms described in these animal studies. Another possible mechanism for the associated muscle tenderness when the TMJ is painful is the concept that protective muscle splinting arises in the muscle tissue around the painful TMJ. The protective muscle splinting is recognized as a central nervous system response to injury and known as an alteration of the muscle activity from the normal levels to protect the threatened part from further injury in the presence of an injury or threat of injury.[19] In the masticatory system, an increase in activity of the jaw elevator muscles occurs during mouth opening when there is pain on motion.[20] A limited mouth opening range is not a pathological condition, although when prolonged, may lead to myalgic symptoms. Pain alleviation in the muscle area should be observed with local anesthesia of the painful TMJ using human subjects or experimental animals to clarify which of the two possible mecha-

nisms are involved. As a result, further experimental studies are necessary to confirm the hypothesis.

Finally, another viewpoint on this topic is the idea that the TMJ pain and the orofacial muscle pains are comorbid problems, and not necessarily demonstrating a particular cause and effect. That is to say, other conditions may thus cause both TMJ and orofacial muscle pain. For instance, it is often argued that nocturnal bruxism has a causal relationship with both TMJ and muscle pain conditions.[21-24] Unfortunately, any attempt to examine this relationship is confounded by the fact that the definition of bruxism varies from study to study. As would be expected, the literature is replete with both positive[25-28] and negative[29,30] suggestions regarding the relationship among them. Further experimental and epidemiologic investigations are therefore required to elucidate these relationships. Such studies may also identify other candidates that have a causal relationship with TMJ and orofacial muscle pain.

REFERENCES

1. Ohlmann B, Rammelsberg P, Henschel V, Kress B, Gabbert O, Schmitter M. Prediction of TMJ arthralgia according to clinical diagnosis and MRI findings. Int J Prosthodont 2006;19:333-8.
2. Ciancaglini R, Radaelli G. The relationship between headache and symptoms of temporomandibular disorder in the general population. J Dent 2001;29:93-8.
3. Graff-Radford SB. Temporomandibular disorders and headache. Dent Clin North Am 2007;51:129-44.
4. Upton LG, Wijeyesakere SJ. The incidence of tinnitus in people with disorders of the temporomandibular joint. Int Tinnitus J 2004;10:174-6.
5. Monaco A, Streni O, Marci MC, Sabetti L, Giannoni M. Convergence defects in patients with temporomandibular disorders. Cranio 2003;21:190-5.
6. Parker MW. A dynamic model of etiology in temporomandibular disorders. J Am Dent Assoc 1990;120:283-9.
7. Sessle BJ, Hu JW. Mechanisms of pain arising from articular tissues. Can J Physiol Pharmacol 1991;69:617-26.
8. Schiffman EL, Anderson GC, Fricton JR, Lindgren BR. The relationship between level of mandibular pain and dysfunction and stage of temporomandibular joint internal derangement. J Dent Res 1992;71:1812-5.
9. Seligman DA, Pullinger AG, Solberg WK. Temporomandibular disorders. Part III: Occlusal and articular factors associated with muscle tenderness. J Prosthet Dent 1988;59:483-9.
10. Wanman A. The relationship between muscle tenderness and craniomandibular disorders: a study of 35-year-olds from the general population. J Orofac Pain 1995;9:235-43.
11. Orsini MG, Kuboki T, Terada S, Matsuka Y, Yatani H, Yamashita A. Clinical predictability of temporomandibular joint disc displacement. J Dent Res 1999;78:650-60.
12. Krogh-Poulsen WG. Management of the occlusion of the teeth. In: Schwartz L, Chayes CM, editors. Facial pain and mandibular function. Philadelphia: W.B. Saunders; 1968. p. 236-79.
13. Curtis AW. Myofascial pain-dysfunction syndrome: the role of nonmasticatory muscles in 91 patients. Otolaryngol Head Neck Surg 1980;88:361-7.
14. Campbell CD, Loft GH, Davis H, Hart DL. TMJ symptoms and referred pain patterns. J Prosthet Dent 1982;47:430-3.
15. Wright EF. Referred craniofacial pain patterns in patients with temporomandibular disorder. J Am Dent Assoc 2000;131:1307-15.
16. Kojima Y. Convergence patterns of afferent information from the temporomandibular joint and masseter muscle in the trigeminal subnucleus caudalis. Brain Res Bull 1990;24:609-16.
17. Ohya A. Responses of trigeminal subnucleus interpolaris neurons to afferent inputs from deep oral structures. Brain Res Bull 1992;29:773-81.
18. Morch CD, Hu JW, Arendt-Nielsen L, Sessle BJ. Convergence of cutaneous, musculoskeletal, dural and visceral afferents onto nociceptive neurons in the first cervical dorsal horn. Eur J Neurosci 2007;26:142-54.
19. Okeson JP, editor. Bell's orofacial pains. 5th ed. Chicago: Quintessence Publishing Co, Inc.; 1995. p. 259-94.
20. Ashton-Miller JA, McGlashen KM, Herzenberg JE, Stohler CS. Cervical muscle myoelectric response to acute experimental sternocleidomastoid pain. Spine 1990;15:1006-12.
21. Kampe T, Edman G, Bader G, Tagdae T, Karlsson S. Personality traits in a group of subjects with long-standing bruxing behaviour. J Oral Rehabil 1997;24:588-93.
22. Molina OF, dos Santos Junior, Nelson SJ, Nowlin T. A clinical study of specific signs and symptoms of CMD in bruxers classified by the degree of severity. Cranio 1999;17:268-79.
23. Manfredini D, Cantini E, Romagnoli M, Bosco M. Prevalence of bruxism in patients with different research diagnostic criteria for temporomandibular disorders (RDC/TMD) diagnoses. Cranio 2003;21:279-85.
24. Baba K, Haketa T, Sasaki Y, Ohyama T, Clark GT. Association between masseter muscle activity levels recorded during sleep and signs and symptoms of temporomandibular disorders in healthy young adults. J Orofac Pain 2005;19:226-31.
25. De Laat A, van Steenberghe D, Lesaffre E. Occlusal relationships and temporomandibular joint dysfunction. Part II: correlations between occlusal and articular parameters and symptoms of TMJ dysfunction by means of stepwise logistic regression. J Prosthet Dent 1986;55:116-21.
26. Seligman DA, Pullinger AG, Solberg WK. The prevalence of dental attrition and its association with factors of age, gender, occlusion, and TMJ symptomatology. J Dent Res 1988;67:1323-33.
27. Schiffman EL, Fricton JR, Haley D. The relationship of occlusion, parafunctional habits and recent life events to mandibular dysfunction in a non-patient population. J Oral Rehabil 1992; 19:201-23.
28. Carlsson GE, Egermark I, Magnusson T. Predictors of signs and symptoms of temporomandibular disorders: a 20-year follow-up study from childhood to adulthood. Acta Odontol Scand 2002;60: 180-5.
29. Droukas B, Lindee C, Carlsson GE. Relationship between occlusal factors and signs and symptoms of mandibular dysfunction. A clinical study of 48 dental students. Acta Odontol Scand 1984;42:277-83.
30. John MT, Frank H, Lobbezoo F, Drangsholt M, Dette KE. No association between incisal tooth wear and temporomandibular disorders. J Prosthet Dent 2002;87:197-203.

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Original article

Gene expression profile of mouse masseter muscle after repetitive electrical stimulation

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Abstract

Purpose: To examine gene expression profile changes in the mouse masseter muscle tissue after repetitive electrical stimulation by using a DNA microarray technique.
Methods: Nine male ICR mice aged 10 weeks were used. Each anesthetized mouse was secured on a platform in a supine position and the masseter muscle tissues on both sides were exposed. Bipolar electrodes were set on the right masseteric fascia to electrically stimulate the masseter muscle (8 V, 10 Hz, 20 ms) for 30 min. After cessation of stimulation bilateral masseter muscle tissues were sampled at 0 h (n = 3), 1 h (n = 3), 2 h (n = 3). Total RNA was isolated from the homogenized muscle tissues and purified mRNA samples (50 μg) were processed and hybridized with microarray slides. Probe arrays were then scanned and analyzed to calculate the signal density. Gene expression profiles were compared at each time point between the right (stimulation side) and left (control side) masseter. When the gene expression levels were different more than 2-fold, the difference was regarded as positive.
Results: Of the 6400 genes assessed, 1733 genes were up-regulated and 515 genes were down-regulated in the stimulation side at least once during the experimental time course. These up- or down-regulated genes were associated with autoimmune/inflammatory disease (28/114), cardiovascular disease (17/61), neuroscience (12/50), apoptosis (27/93), diabetes/obesity (9/28), signal transduction (66/250) and others. 28 genes were up-regulated and 25 genes were down-regulated at all time points.
Conclusions: Dramatic gene expression changes were induced by the repetitive electrical muscle stimulation in mouse masseter.
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Keywords: Masseter muscle; Gene expression; Electrical stimulation; Mouse; Microarray

1. Introduction

For many years, it has been argued that oral parafunctions (e.g., tooth clenching or grinding) causes fatigue and pain in masticatory muscles [1–5]. However, since scientific evidence linking oral parafunctions and masticatory muscle pain is still lacking, the premise is still controversial. In order to clarify the relation between parafunction and muscle pain, it is important to know the local biological phenomena induced by muscle hyperactivity. However, local biological phenomena produced during or after muscle contraction in masticatory muscles have been rarely investigated yet.

Regarding the limb muscles, several attempts have been carried out to observe protein or gene expression changes of some candidate molecules during and after experimentally induced muscle activities. Chen et al. (2003) investigated the effect of eccentric exercise on the transcriptome of skeletal muscle in male human subjects who performed 300 concentric contractions with one leg and 300 eccentric contractions with opposite leg [6]. Muscle biopsies were taken from both legs at 4–8 h after exercise and expression was profiled by using microarray with 12,000 genes. The results of their study revealed the great inflammatory responses (chemokine (C–C motif) ligand-2, C/EBP delta, and IL-1 receptor) and vascular remodeling (tenescin C and lipocortin II). While the response is

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